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Enhancing lipophilicity as a strategy to overcome resistance against platinum complexes?

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ABSTRACT

Decreased influx represents one of the major resistance mechanisms of platinum complexes. In order to address the question if this mechanism of resistance can be overcome by enhancing the lipophilicity of platinum complexes, we investigated the influence of lipophilicity on cellular accumulation and cytotoxicity in a panel of oxaliplatin analogues with different carrier ligands. Cellular accumulation, DNA platination and cytotoxicity were measured in a cisplatin-sensitive and -resistant ovarian carcinoma (A2780/A2780cis) and in an oxaliplatin-sensitive and -resistant ileocecal colorectal adenocarcinoma (HCT-8/HCT-8ox) cell line pair. Platinum concentrations were determined by flameless atomic absorption spectrometry or adsorptive stripping voltammetry. Passive diffusion represented the main influx mechanism of oxaliplatin analogues during the first minutes of incubation as indicated by a correlation between lipophilicity and early influx rate. Afterwards, the predominant influx mechanism was lipophilicity-independent. More lipophilic complexes showed a reduced cytotoxic activity, although the early influx rate was increased. The resistance profiles of the two cell line pairs were found to be different: HCT-8ox cells were less resistant against more lipophilic complexes, whereas A2780cis cells exhibited a comparable degree of resistance against all investigated compounds. However, the reduction in resistance factor of HCT-8ox cells cannot be explained by increased influx suggesting that other resistance mechanisms are circumvented upon exposure to more lipophilic compounds. Though resistance against more lipophilic platinum complexes analogues is lower we conclude that enhancing lipophilicity is not a successful strategy to overcome platinum resistance as higher lipophilicity is also associated with lower cytotoxic activity.

1. Introduction

Platinum complexes are routinely used in clinical practice for treatment of testicular, ovarian and other solid tumors. However, the therapeutic outcome of platinum-based chemotherapy can be impaired by intrinsic or acquired resistance. Resistance is one of the major limitations of platinum anticancer chemotherapy and is the consequence of multifactorial events. Until now, several involved mechanisms have been identified [1–4]. Among those, reduced drug accumulation is the most frequently observed phenomenon in platinum-resistant cell lines, which may be due to increased efflux, reduced influx or both [5]. In our work, we have focused on the influx in order to develop novel platinum complexes which will no longer be reliant on those influx mechanism(s) responsible for reduced cellular accumulation.

Gately and Howell postulated that the influx of platinum complexes is mediated by passive diffusion and by facilitated diffusion through a gated channel [5]. Lately this model was broadened to include not only gated channels but also facilitated and active transport mechanisms involving a number of transport proteins [6]. The contribution of the copper transporter CTR1 and the organic cation transporters OCT1-3 has frequently been discussed [6,7]. Recently we characterized the cisplatin-sensitive and -resistant ovarian carcinoma cell line pair A2780/A2780cis regarding influx and efflux of cisplatin [8]. We compared the expression of copper transporters, intracellular platinum accumulation, DNA platination and cytotoxicity of cisplatin in sensitive and resistant ovarian carcinoma cells. We found a lower intracellular platinum accumulation and significantly lower degree of DNA platination in the resistant variant compared to the respective sensitive cell line, whereas there were no differences in efflux rate.

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Resistant cells expressed lower levels of CTR1 (1.5–1.8-fold) than their sensitive counterparts. These results highlight the relevance of the influx of platinum complexes for sensitivity as there is a clear relationship between CTR1 expression, platinum accumulation, DNA platination and cytotoxicity of cisplatin in this cell line pair [8].

Depending on the influx mechanism, different physicochemical properties of platinum complexes may influence cellular accumulation. Passive diffusion mainly depends on lipophilicity and charge, whereas the reactivity of platinum complexes has been discussed as a predominant factor affecting transport by proteins, for example CTR1. It is assumed that platinum complexes bind to methionine-rich domains of CTR1 and enter the cell either via a stabilized channel or by endocytosis as a receptor complex [9–12]. Information about the factors which influence the uptake of platinum complexes by the organic cation transporters OCT1-3 is still limited.

In this study we investigated the effect of lipophilicity of platinum complexes on cellular accumulation and cytotoxicity. It has been suggested that increasing lipophilicity may help overcome accumulation defects and consequently enhance cytotoxicity in resistant cell lines [13]. Moreover, lipophilicity was shown to correlate with cytotoxic activity [14–16]. In other studies a strong relationship between intracellular platinum concentrations and lipophilicity of platinum complexes was demonstrated [13,17–19]. Most of the reported investigations have been carried out with platinum(IV) complexes or with a panel of structurally very different platinum complexes. In order to draw conclusions regarding the influence of lipophilicity on cellular accumulation, other factors like oxidation state or reactivity should be comparable between the complexes.

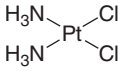
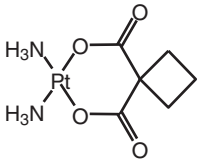
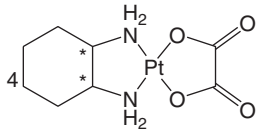
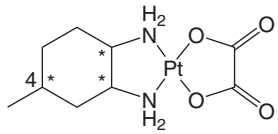
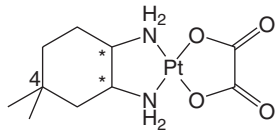
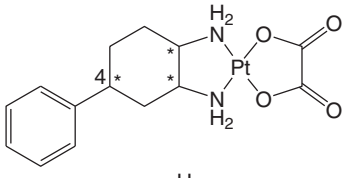
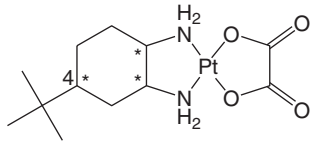
This paper describes the first systematic investigation of the relationships between lipophilicity, cellular accumulation and cytotoxic activity of a new class of oxaliplatin derivatives with different substituents at position 4 of the cyclohexane ring in two different sensitive/resistant cell line pairs (Table 1). We used the above-mentioned well characterized sensitive/resistant ovarian carcinoma cell line pair A2780/A2780cis and the ileocecal colorectal adenocarcinoma cell line pair HCT-8/HCT-8ox. The A2780cis (resistant) cell line acquired resistance after cisplatin treatment, whereas the HCT-8ox (resistant) cell line was obtained after oxaliplatin exposure. Both cell lines were chosen in order to distinguish between acquired cisplatin and oxaliplatin resistance.

2. Experimental

2.1. Chemicals

Synthesis of the platinum complexes was carried out according to the procedures previously published [20–22]. Carboplatin, cisplatin and oxaliplatin were obtained from Sigma-Aldrich, Steinheim, Germany.

Table 1
Structure, stereochemistry and lipophilicity of the investigated platinum complexes (n.a., not applicable, *, chiral center).

	Complex	Stereochemistry at C(4)	Structure	log P
Cisplatin	*cis*-diamminedichloroplatinum(II)	n.a.		−2.53
Carboplatin	*cis*-diammine(1,1-cyclobutane-dicarboxylato)platinum(II)	n.a.		−2.30
Oxaliplatin	(*trans*-R,R-cyclohexane-1,2-diamine) oxalatoplatinum(II)	n.a.		−1.76
4	(SP-4-3)-(4-methyl-*trans*-cyclohexane-1,2-diamine) oxalatoplatinum(II)	Equatorial		−1.28
5	(SP-4-3)-(4,4-dimethyl-*trans*cyclohexane-1,2-diamine) oxalatoplatinum(II)	n.a.		−0.68
6	(SP-4-3)-(4-phenyl-*trans*-cyclohexane-1,2-diamine) oxalatoplatinum(II)	91% axial		−0.28
7	(SP-4-3)-(4-tert-butyl-*trans*-cyclohexane-1,2-diamine) oxalatoplatinum(II)	Equatorial		0.42

DMSO was purchased from Riedel-de Haën, Seelze, Germany, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) from AppliChem, Darmstadt, Germany, and nitric acid 65% (Suprapur) from Merck, Darmstadt, Germany. 2′-deoxyguanosine 5′-monophosphate sodium salt (dGMP), 2′-deoxyadenosine 5′-monophosphate sodium salt (dAMP), thymidine 5′-monophosphate disodium salt (TMP), 1-octanol, RPMI-1640™ medium, L-glutamine solution (200 mM), penicillin-streptomycin solution (10,000 I.E./mL, 10 mg/mL), fetal calf serum and trypsin–EDTA solution (0.5 g porcine trypsin and 0.2 g EDTA in 100 mL) were obtained from Sigma-Aldrich, Steinheim, Germany, and boric acid, monosodium phosphate and sodium chloride from Fluka Chemie, Neu-Ulm, Germany. Ultrapure water was obtained using a Purelab Plus™ system (ELGA Labwater, Celle, Germany).

2.2. Determination of log P

The log P values were determined according to the OECD guideline using the shake-flask method [23]. Weighted amounts of platinum complexes were partitioned between an aqueous and 1-octanol phase. Platinum concentrations in the aqueous phase were measured by flameless atomic absorption spectrometry. Based on these results, the partition coefficients were calculated.

2.3. Capillary electrophoresis

For the investigation of the reactivity of platinum complexes towards the nucleotides dAMP and dGMP, we modified and validated a method previously described [24]. Briefly, 150 μM of each platinum complex was incubated with 150 μM dGMP and 150 μM dAMP under physiological conditions (4 mM NaH_2PO_4, 4 mM NaCl, pH 7.4, 37 °C) up to 72 h. The samples were collected after 0, 24, 48 and 72 h of incubation ($n = 3$). To enhance the precision of the nucleotide determination, 4 μL internal standard (TMP, 3 mM in buffer containing 4 mM NaH_2PO_4, 4 mM NaCl, pH 7.4) was added to 100 μL sample. The measurement was carried out on a P/ACE™ 5510 instrument controlled by the P/ACE™ station software version 1.21 (Beckman Coulter™, Fullerton, USA). An uncoated capillary (75 μm ID, effective separation length 57 cm) was assembled in the P/ACE™ cartridge. The capillary was thermostated at 19 °C. Injections were performed at 5 psi for 5 s. A voltage of 20 kV was applied for all experiments. Detection was performed using UV absorption at 254 nm. Prior to each analysis the capillary was flushed with the running buffer (400 mM borate buffer, pH 9.1) for 1 min. After each analysis the capillary was rinsed with sodium hydroxide (0.1 M) for 1 min, followed by ultrapure water for 1 min.

2.4. Platinum analysis

The platinum concentrations were measured by flameless atomic absorption spectrometry using a validated modification of the procedure described by Kloft et al. [25]. In brief, an atomic absorption spectrometer (SpectrAA™ Zeeman 220; Varian, Darmstadt, Germany) equipped with a graphite tube atomizer (GTA 100), a programmable sample dispenser (PSD 100) and a platinum hollow cathode lamp (UltrAA™ lamp) were used. The temperature program included an ashing step at 1300 °C and an atomization step at 2700 °C. The lower limit of quantification (LLOQ) was 1 ng/mL.

Samples with platinum concentrations below the LLOQ of flameless atomic absorption spectrometry were analyzed using a validated adsorptive stripping voltammetry method [26]. With this method the detection limit could be reduced to 0.4 pg/mL. In brief, after drying the samples were mineralized by a high-pressure asher (HPA, Kürner, Rosenheim, Germany) [27]. Platinum was then quantified by adsorptive stripping voltammetry using a Metrohm Polarecord 693 VA Processor with 694 VA Stand (Metrohm, Herisau, Switzerland). The standard addition technique was used to determine the platinum concentrations [26].

2.5. Cell lines and cell culture

The human ovarian carcinoma cell lines A2780 and A2780cis (European Collection of Cell Cultures, United Kingdom) [28] and the human ileocecal colorectal adenocarcinoma cell lines HCT-8 and HCT-8ox (kindly provided by Dr. M. Heim, University of Essen, Germany) were used in the study. The resistant variants were obtained by incubation with stepwise increasing cisplatin or oxaliplatin concentrations, respectively. Backups of all cells were frozen with 10% DMSO. A2780/A2780cis and HCT-8/HCT-8ox cells were cultivated in RPMI-1640™ medium after adding 10% fetal calf serum, 100 I.E./mL penicillin and 0.1 mg/mL streptomycin. In the case of A2780/A2780cis 0.6 mM L-glutamine was added. The cells were cultivated as monolayers at 37 °C in a humidified atmosphere containing 5% CO_2. Every 12 passages, a new backup of cells was thawed to ensure that resistance mechanisms were unchanged during long-time cultivation. The maintenance of resistance was checked at the first and at the last passage using an MTT-based assay.

2.6. Platinum accumulation

In order to characterize the influx of the platinum compounds, 2×10^6 cells were incubated with 100 μM platinum complex up to 2 h. After certain time points the medium was discarded quickly and cells were washed with 1 mL ice-cold PBS (phosphate buffered saline, pH adjusted to 7.4). Then cells were trypsinized, resuspended in fresh drug-free medium and centrifuged for 1 min at 4 °C and 1520 *g*. The supernatant was discarded and the pellet was washed twice in 1 mL ice-cold PBS. After centrifugation for 1 min at 18,620 *g* the supernatant was discarded again and the cell pellet was frozen at −20 °C until further analysis. Immediately after thawing the cells were lyzed with concentrated nitric acid for 1 h on a water bath at 80 °C. Then intracellular platinum concentrations were measured by flameless atomic absorption spectrometry or adsorptive stripping voltammetry. Platinum concentrations were calculated in relation to the mean cell volume (as measured with Casy™1 cell counter, Schärfe System, Reutlingen, Germany).

In order to characterize the efflux of platinum, 2×10^6 cells were incubated with 100 μM platinum complexes for 2 h. A part of the cells was collected as described above. The other part of the cells was washed two times with 1 mL PBS and incubated for 2, 5, 10, 60 and 120 min with drug-free medium. Afterwards, the cells were collected as described above.

2.7. DNA platination

5×10^6 cells were incubated with 100 μM platinum complex up to 4 h and subsequently washed with 1 mL ice-cold PBS. Afterwards, cells were trypsinized, resuspended in fresh drug-free medium and centrifuged for 1 min at 4 °C and 1520 *g*. The supernatant was discarded and the pellet was washed twice in 1 mL ice-cold PBS. After centrifugation for 1 min at 18,620 *g* the supernatant was discarded again and the cell pellet was frozen at −20 °C until further analysis.

DNA platination was determined using a previously published method adapted to tumor cells [29]. After isolation with solid-phase extraction (QIAmp®, Qiagen, Hilden, Germany) DNA concentrations were measured by UV photometry and platinum concentrations by flameless atomic absorption spectrometry or adsorptive stripping voltammetry. Based on these results the platinum–nucleotide ratio [Pt atoms/10^6 nucleotides] was calculated as a measure of the extent of DNA platination. The platinum–nucleotide ratio reflects the net rate of adduct formation and repair.

2.8. Membrane preparation

In order to determine the fraction of platinum complex bound to membranes, 10^7 cells were incubated with 100 μM platinum complex for 2 h and subsequently washed with 1 mL ice-cold PBS. Afterwards, cells were trypsinized, resuspended in fresh drug-free medium and centrifuged for 1 min at 4 °C and 5000 *g*. The supernatant was discarded and the pellet was washed again with 1 mL ice-cold PBS. After centrifugation for 1 min at 5000 *g* the supernatant was discarded. The pellet was resuspended in ice-cold PBS buffer and the cells were disrupted using a Polytron homogenizer (Kinetmatica AG, Basel, Switzerland). The homogenate was centrifuged for 10 min at 2 °C and 40,000 *g*. The supernatant contained the cytosolic platinum. It was stored at −20 °C until further analysis. The pellet was washed again two times with ice-cold PBS followed by centrifugation for 10 min at 2 °C and 40,000 *g* [30]. After the washing steps the pellet consisting of the membrane-bound platinum was resuspended in ultrapure water and stored at −20 °C until further analysis. The samples of the cytosolic and membrane-bound platinum were measured by flameless atomic absorption spectrometry. Based on these results the percentage of platinum in the membrane was calculated.

2.9. Cytotoxicity assay

Cytotoxicity of the platinum complexes was assessed using an MTT-based assay [31]. Briefly, cells were plated in 96-well microtiter plates (approximately 5000–10,000 cells/well) and allowed to attach. Then medium was removed. Stock solutions of platinum complexes in ultrapure water (0.8 mM) were diluted in medium and nine subsequent dilutions were added to the cells in triplicate (100 μL/well). After 72 h of incubation, 20 μL of a 5 mg/mL MTT solution (in PBS) was added to each well, and the cells were incubated at 37 °C for about 1 h. Subsequently, medium was discarded and 100 μL DMSO was added to dissolve the formazan crystals. Absorbance of the converted dye was measured at 570 nm with background subtraction at 690 nm using a Multiskan Ascent® microtiter plate reader (Thermo Fisher Scientific, Langenselbold, Germany). The results were analyzed and the pEC_{50} values ($pEC_{50} = -\log EC_{50}$, EC_{50} is the drug concentration that produces 50% of the maximum possible response) were estimated with the GraphPad Prism™ analysis software package (GraphPad Software, San Diego, USA) using non-linear regression (sigmoidal dose response, variable slope). The resistance factor (RF) was calculated by dividing the EC_{50} in the resistant variant by the EC_{50} in the respective sensitive cell line.

2.10. Statistical analysis

Although the results of the Kolmogorov–Smirnov test suggested a Gaussian distribution of the data from the cell culture experiments, the sample number was too small to exclude a non-Gaussian distribution. Therefore, we chose the median as measure of central tendency. Consequently, differences were analyzed using the non-parametric Mann–Whitney test or the Kruskal–Wallis test, as appropriate. Correlation analyses were performed using the non-parametric Kendall tau rank correlation. P values of ≤ 0.05 were considered significant. By contrast, the EC_{50} values are generally accepted to be log-normal distributed. In this case, it was regarded as appropriate to calculate mean pEC_{50} values.

3. Results

3.1. Platinum complexes

The introduction of substituents at position 4 of the cyclohexane ring creates an additional chiral center. Thus, the compounds **4**, **6** and **7** contain alkyl groups in equatorial and/or in axial position. **4** and **7** feature exclusively equatorial substitution, and **6** is mainly axially substituted (Table 1) [21,22,32].

3.2. Determination of log P

The results of the log P determination are shown in Table 1. As expected lipophilicity of the compounds increases in the following order: cisplatin<carboplatin<oxaliplatin<**4**<**5**<**6**<**7**.

3.3. Reactivity towards nucleotides

During the incubation of the platinum complexes with nucleotides, platinum–nucleotide adducts were formed. The reactivity of the platinum complexes was determined based on the decrease in the amount of free nucleotide (Fig. 1A and B). A faster decrease in the concentration of dGMP compared to the concentration of dAMP was observed in the presence of all tested platinum complexes. Oxaliplatin and its analogues showed a comparable reactivity towards the nucleotides. For comparison, the reactivity of carboplatin and cisplatin was also determined. Cisplatin turned out to be the most reactive and carboplatin the least reactive compound.

3.4. Platinum accumulation — influx

During the incubation experiments the platinum accumulation within the cells was measured. The platinum accumulation reflects the net rate of influx and efflux. Because of the infeasibility to determine each process separately, conditions were chosen to favor

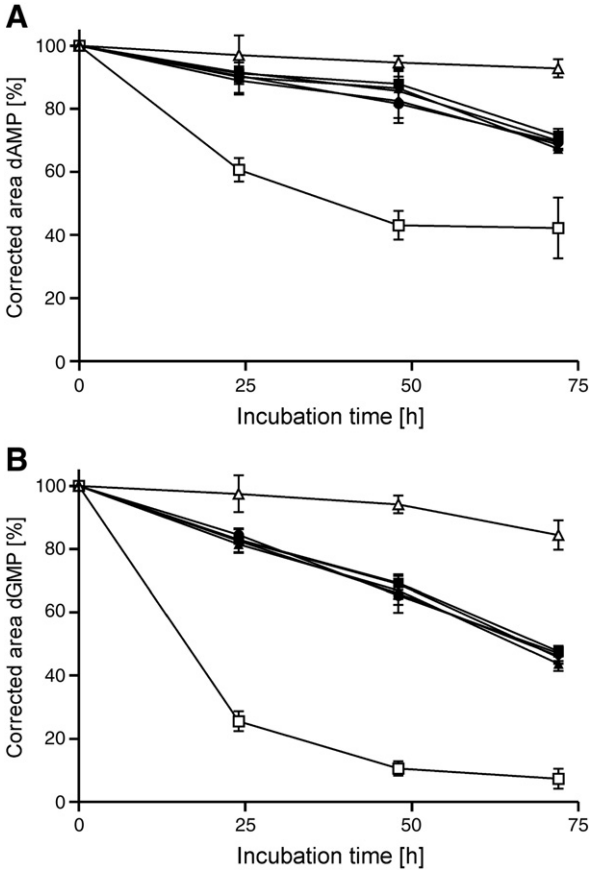


Fig. 1. Reduction of the corrected peak area of the nucleotides (A) dAMP and (B) dGMP during incubation with 150 μM platinum complex: cisplatin (□), carboplatin (△), oxaliplatin (◆), **4** (●), **5** (▲), **6** (▼), and **7** (■) (n = 3, mean ± SD).

one of the processes. Thus, a high extracellular concentration of platinum complex was used to boost influx.

In general, the ovarian carcinoma cell line pair (Fig. 2A, B) showed higher platinum accumulation than the ileocecal colorectal adenocarcinoma cell line pair (Fig. 2C, D). During the incubation with oxaliplatin and its analogues up to 2 h, platinum accumulation exhibited an approximately linear increase (except **7**). A reduction of the platinum accumulation in the resistant cell lines was found for all compounds except **7**. This highly lipophilic compound showed a higher platinum accumulation in both resistant cell lines after 2 h of incubation compared to the respective sensitive cell line.

For comparison of the concentration–time profiles of the different platinum complexes we differentiated between the early and late influx rate. The influx rate within 10 min of incubation was used as an indicator for the early influx (early influx rate). Due to the approximately linear increase of the platinum accumulation between the 10th and 120th min observed in both cell line pairs, a linear regression of the median concentration–time profile was performed for each compound. The slope obtained reflected the influx rate in the late influx phase (late influx rate).

The relationship between lipophilicity and the early influx rate is shown in Fig. 3. Good to relatively strong correlations were found in all cell lines ($0.80 \leq r \leq 1.00$). These findings suggest an influence of lipophilicity on the early influx of the platinum complexes. Moreover, already after 10 min of incubation a reduced influx rate of some complexes in the resistant cell lines was observed. The difference was statistically significant for oxaliplatin and **4** in both resistant cell lines and for **7** in HCT-8ox cells.

In Fig. 4 the calculated late influx rates of the complexes are displayed. No significant correlations between lipophilicity and late influx rates were found in the investigated cell lines except for A2780cis cells ($r = 0.80$, $p = 0.05$). Late influx rates of a given compound in resistant cells were lower compared to the respective sensitive cell line. The only exception was **7** exhibiting a higher late influx rate in both resistant cell lines.

3.5. Platinum accumulation — efflux

Investigation of the efflux was important for two reasons: Firstly, the reduced platinum accumulation in the resistant cell lines may be a result of increased efflux and secondly, efflux may contribute to the striking differences in platinum accumulation after incubation with **7** as compared to oxaliplatin (Fig. 2). Therefore, efflux rate of oxaliplatin as the least lipophilic compound and **7** as the most lipophilic compound was assessed by measuring the platinum accumulation after incubation with the complexes and subsequent incubation with drug-free medium (Fig. 5). In both ovarian carcinoma cell lines rapid oxaliplatin efflux in the first 10 min of incubation with drug-free medium and a subsequent efflux plateau were observed, with no major differences between the sensitive and the resistant cell lines (Fig. 5A). Efflux of **7** from A2780 and A2780cis cells was much slower compared to oxaliplatin in both sensitive and resistant cells.

In the ileocecal colorectal adenocarcinoma cell line pair (Fig. 5B), oxaliplatin efflux was slow, only after 1 h the efflux rate seemed to be enhanced. The efflux of **7** was fast only in the first minutes. Neither

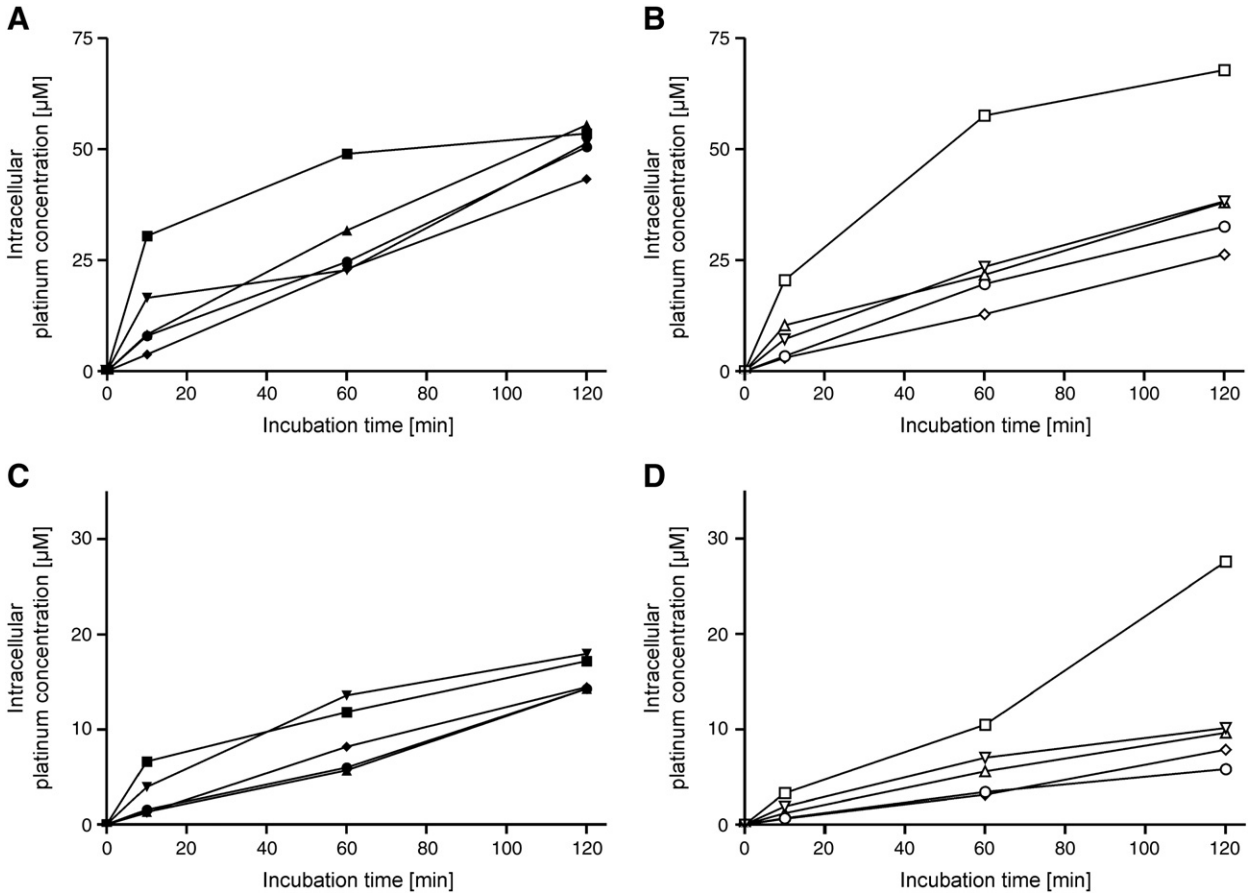


Fig. 2. Intracellular platinum concentration in (A) A2780, (B) A2780cis, (C) HCT-8 and (D) HCT-8ox cells during incubation with 100 µM platinum complex: oxaliplatin (♦), **4** (●), **5** (▲), **6** (▼), and **7** (■) in the sensitive cell lines and oxaliplatin (◊), **4** (○), **5** (△), **6** (▽), and **7** (□) in the resistant cell lines ($n = 3$–18, median, for purposes of clarity a measure of variability is not shown).

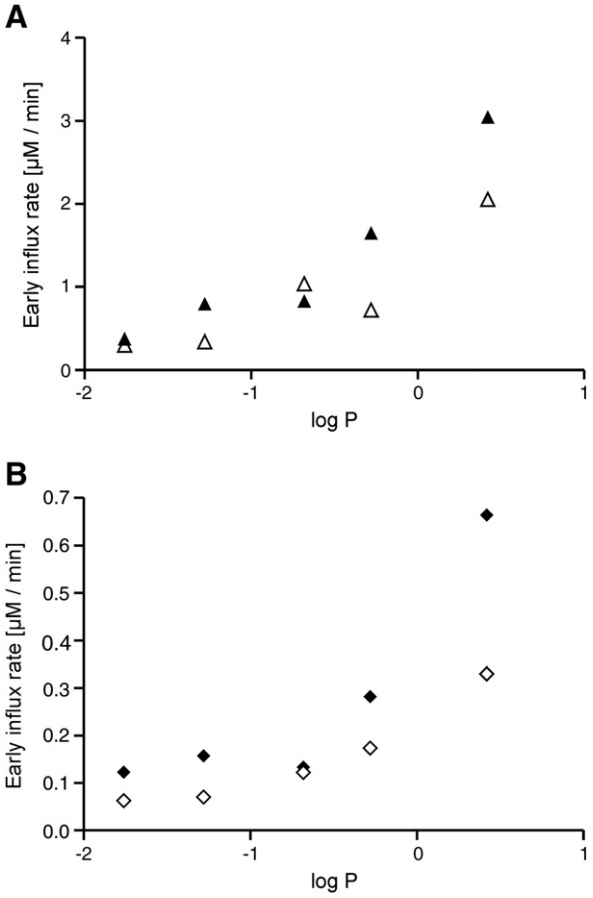


Fig. 3. Dependence of the early influx rate on the lipophilicity of oxaliplatin and the platinum complexes **4**–**7** in (A) A2780 (▲), A2780cis (△), (B) HCT-8 (♦) and HCT-8ox (◊) cells after incubation with 100 µM platinum complex for 10 min (n = 3–18, median).

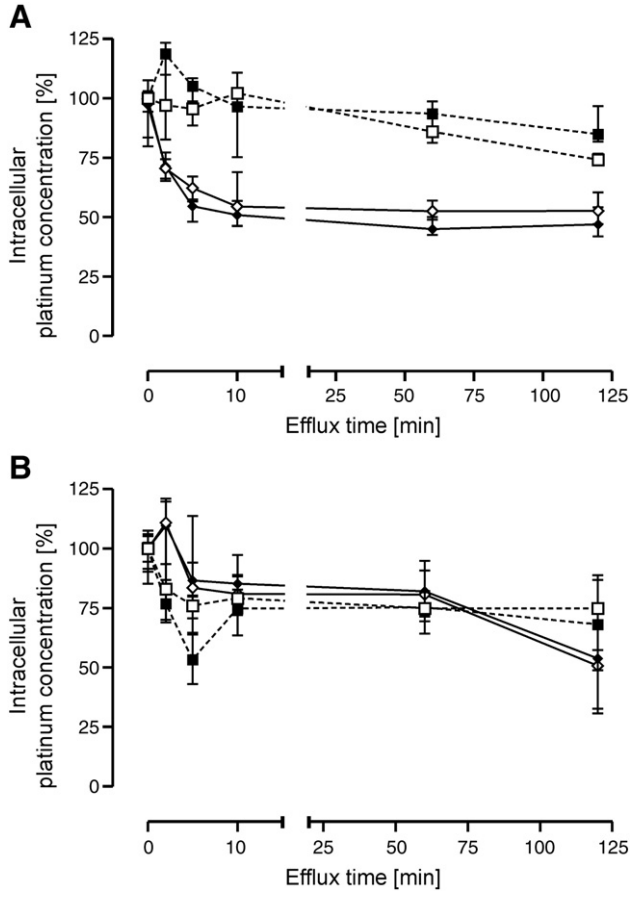


Fig. 5. Intracellular platinum concentration (%) in (A) A2780/A2780cis and (B) HCT-8/HCT-8ox cells after 2 h of incubation with oxaliplatin (♦, sensitive cells, ◊, resistant cells) and complex **7** (■, sensitive cells, □, resistant cells) and subsequent incubation with drug-free medium (n = 6, median ± interquartile range). The median platinum concentration at the end of the 2 h incubation period was set to 100%.

the efflux of oxaliplatin nor the efflux of **7** differed noteworthy between HCT-8 and HCT-8ox cells.

These results suggest that differences in efflux of oxaliplatin and **7** between sensitive and resistant cells are negligible in both cell line pairs. Therefore, the different cellular accumulation of both compounds in the resistant cell lines seems to be due to differences in influx rather than efflux. The reduced efflux of **7** compared to oxaliplatin in the ovarian carcinoma cell line pair, might explain the higher accumulation of this compound, but cannot explain the higher accumulation in the resistant cell lines compared to the sensitive cell lines.

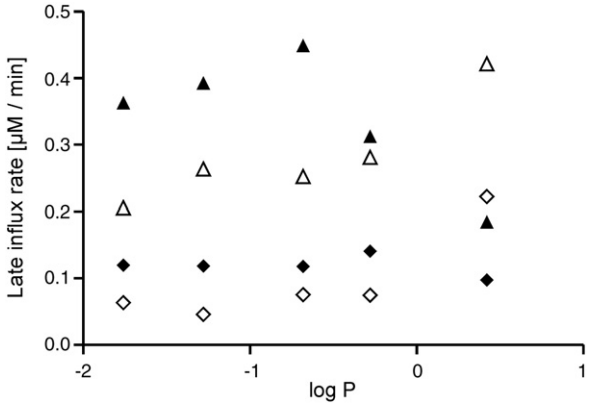


Fig. 4. Dependence of the late influx rate on the lipophilicity of oxaliplatin and the platinum complexes **4**–**7** in A2780 (▲), A2780cis (△) HCT-8 (♦) and HCT-8ox (◊) cells after incubation with 100 µM platinum complex (n = 1–6, median).

3.6. Cytotoxicity

The sensitivity of each cell line to oxaliplatin and its analogues is presented in Table 2. Except for oxaliplatin all complexes showed a higher cytotoxicity in the ovarian carcinoma cell line pair. The low cytotoxicity of the most lipophilic complex **7** compared to the other complexes was remarkable.

The determined pEC_{50} values were inversely correlated with the lipophilicity of the complexes. A strong correlation was found for all investigated cell lines ($-0.80 \leq r \leq -1.00$). Increased lipophilicity of the oxaliplatin analogues is hence associated with lower cytotoxicity.

In order to investigate the possibility to overcome resistance using more lipophilic platinum complexes, we also tested the correlation between lipophilicity and the resistance factor. No relationship was found in the ovarian carcinoma cell line pair, but a strong inverse correlation was observed in the ileocecal colorectal adenocarcinoma cell line pair ($r = -1.00$, $p < 0.01$).

3.7. DNA platination

In order to address the question to which extent oxaliplatin and **7** (as the least and the most lipophilic complex, respectively) form DNA adducts, we analyzed the DNA platination in both cell line pairs. For both oxaliplatin and **7**, no significant differences in the DNA adduct formation were found between A2780 and A2780cis cells.

Table 2
pEC_{50} values and resistance factors (RF) of oxaliplatin and the platinum complexes **4**–**7** in the A2780 and HCT-8 cell line pair (n = 3, mean ± SE).

		Oxaliplatin	**4**	**5**	**6**	**7**
pEC_{50}	A2780	5.39 ± 0.03	5.42 ± 0.09	5.23 ± 0.04	4.74 ± 0.07	4.59 ± 0.09
	A2780cis	4.87 ± 0.09	4.71 ± 0.09	4.71 ± 0.08	4.41 ± 0.10	4.00 ± 0.12
RF		3.3	5.1	3.3	2.1	4.1
pEC_{50}	HCT-8	6.11 ± 0.03	5.31 ± 0.09	5.19 ± 0.05	4.51 ± 0.06	4.14 ± 0.05
	HCT-8ox	5.11 ± 0.03	4.45 ± 0.01	4.58 ± 0.06	4.16 ± 0.01	4.10 ± 0.05
RF		10.0	7.3	4.0	2.2	1.1

After incubation with oxaliplatin 56.2 (7.3) Pt atoms were bound to 10^6 nucleotides in A2780 cells and 51.4 (4.5) in A2780cis cells (n = 3; medians and interquartile ranges). After exposure to **7** 22.2 (9.3) and 17.6 (12.0) Pt atoms/10^6 nucleotides were measured in A2780 and A2780cis cells, respectively, which was significantly lower compared to oxaliplatin ($p = 0.05$ for both cell lines). Oxaliplatin formed on average 2.5-fold (A2780) and 2.9-fold (A2780cis) more platinum-DNA adducts than **7**.

A different situation was observed in the ileocecal colorectal adenocarcinoma cell line pair (Pt atoms/10^6 nucleotides after oxaliplatin exposure in HCT-8 cells: 36.3 (17.0), in HCT-8ox cells: 15.1 (3.5); after incubation with **7** in HCT-8 cells: 7.8 (3.6), in HCT-8ox cells 4.9 (0.7); n = 6, median (IQR)). A 2.4-fold higher level of oxaliplatin-DNA adducts was found in HCT-8 as compared to HCT-8ox ($p<0.01$), whereas the extent of DNA platination by **7** was only 1.6-fold higher and did not differ significantly between HCT-8 and HCT-8ox cells. Comparison of DNA platination in the presence of oxaliplatin and **7** shows that oxaliplatin formed on average 4.6-fold more adducts in HCT-8 ($p<0.01$) and 3.1-fold more adducts in HCT-8ox cells ($p<0.01$).

3.8. Membrane preparation

Due to the fact, that the accumulation of **7** was increased compared to oxaliplatin while the formation of platinum-DNA adducts was decreased, we analyzed the relationship between the amount of platinum in the cell membrane and in the cytosol. The lipophilic complex **7** accumulated to a slightly higher extent in the membrane of A2780 and A2780cis cells compared to oxaliplatin (% platinum fraction in the membrane after oxaliplatin exposure in A2780 cells: 24.1 (9.4), in A2780cis cells: 28.2 (18.9); after incubation with **7** in A2780 cells: 36.8 (9.0), in A2780cis cells: 38.6 (41.9); n = 6, mean (IQR)). However, the difference was not statistically significant.

4. Discussion

4.1. Cellular accumulation

The primary objective of this investigation was to reveal whether it is possible to overcome one of the main oxaliplatin resistance mechanisms – a reduced influx – by enhancing the lipophilicity of the complex. For this purpose, the influence of lipophilicity on the influx kinetics of oxaliplatin analogues was assessed in different tumor cell lines. Reactivity towards nucleophiles has been reported to play a major role in the influx, especially in the influx mediated by CTR1 [10,33]. Despite possessing the same leaving group, differences in the reactivity of the investigated platinum complexes cannot be excluded due to altered steric hindrance. However, all investigated oxaliplatin analogues showed a comparable reactivity. Therefore, we assume that the differences in the cellular accumulation are mainly related to the different lipophilicities of the platinum complexes. Nonetheless, an influence of other so far unknown properties of platinum complexes with relevance for influx cannot be excluded.

Within the first minutes of incubation a correlation between log P values and the influx rate was observed, suggesting a lipophilicity-dependent early influx. Most likely passive diffusion of the complexes in or across the plasma membrane dominates in the early influx phase. Passive diffusion should still proceed in the late influx phase, however with a reduced rate because of the reduced concentration gradient. In fact, the late influx rate was lower than the early influx rate, but no longer determined by lipophilicity. We therefore suppose that reactivity mainly influences the late influx rate. This may correspond to a reactivity-dependent binding of the platinum complexes to transport proteins, e.g. CTR1 or OCT1-3 [5,6,34]. In both resistant cell lines a reduction of the influx rate, even in the early influx phase, was found. As previously described for the ovarian carcinoma cell line this may be associated with the reduced expression of CTR1 [8].

In contrast, the late influx rate of the most lipophilic platinum complex, **7**, was increased in both resistant cell lines compared to their sensitive counterparts while the efflux of **7** was not altered. Therefore, the higher accumulation may be a result of an increased influx. If the lipophilicity of this compound was the only reason for the increased late influx rate, the influx rate in the sensitive cell lines would also be increased, which was not the case. One may speculate that the biophysical properties of the plasma membrane of the resistant cells are altered leading to a favored transport of lipophilic complexes in or across the membrane. Changes in the membrane composition of the resistant cell lines have been associated with reduced influx [5,6]. In a cisplatin-resistant cell line a lower membrane fluidity due to a tighter packing of phospholipid molecules, an altered dynamic character of lipid molecules and a lower index of unsaturated fatty acids were observed [35]. On the contrary, more “fluid” plasma membranes were found in another cisplatin-resistant cell line [36]. The changes appear to be cell-line specific and their contribution to resistance has been discussed controversially.

4.2. Cytotoxicity

The increased accumulation of the more lipophilic complexes found in each cell line leads to the question whether it is possible to increase the cytotoxic potency of a platinum complex in a given cell line by increasing the lipophilicity. Given the observed differences in the accumulation of the platinum compounds in the early influx phase, an increase in cytotoxicity with increasing lipophilicity could be expected. This has already been described for other platinum complexes [15,16,37]. Surprisingly, an inverse correlation between lipophilicity and cytotoxicity was found in all cell lines. A lower cytotoxic potency of the oxaliplatin derivatives with bulky substituents at position 4 of the cyclohexane ring was previously reported in different human cancer cell lines [21,22]. In order to understand the reduced cytotoxic potency of the more lipophilic complexes we investigated the intracellular fate of oxaliplatin and **7** as the least and the most lipophilic compounds in more detail.

The reduced cytotoxicity of **7** was accompanied by a reduced DNA platination compared to oxaliplatin although the cellular accumulation of this complex was higher. On the one hand, we cannot completely exclude changes in reactivity of the platinum complexes with bulky substituents once they have entered the cell. The reduced DNA platination might be a result of a reduced reactivity of **7** towards

DNA compared to the reactivity towards single nucleotides. On the other hand, this observation might suggest, that **7** is trapped in the cytoplasm, either by binding to nucleophiles and therefore inactivation or by sequestration in subcellular compartments [38–41]. There is already evidence for sequestration of lipophilic platinum complexes. A platinum compound with a lipophilic anthraquinone carrier ligand was found to accumulate in lysosomes. It has been discussed that the amphiphilicity of this platinum complex leads to partitioning in the plasma membrane without diffusion through the membrane followed by internalization by endosomes and accumulation in lysosomes [38]. Partitioning of **7** in the plasma membrane is further strengthened by the observation that the lipophilic compound **7** accumulates to a slightly higher extent in the membrane than oxaliplatin. Mitochondria may represent another cellular organelle capable of sequestration of platinum complexes. The increased mitochondrial membrane potential in tumor cells compared to normal epithelial cells could result in an increased accumulation of lipophilic cations [42]. According to a predictive model, strong bases and permanent cations with intermediate lipophilicity (log P between −2 and 2 with an optimum at 0) tend to accumulate in mitochondria of tumor cells [43]. Although there is some evidence of an involvement of lysosomes and mitochondria in sequestration of platinum compounds, the exact subcellular localization of **7** needs further clarification.

To summarize, enhancing lipophilicity of oxaliplatin by introducing substituents to the DACH carrier ligand failed to enhance cytotoxicity in the investigated cell lines.

4.3. Resistance

In our previous study we observed a correlation between the expression of CTR1, intracellular platinum concentration, DNA platination and cell death [8]. Resistance of A2780cis cells to cisplatin was mainly based on a reduced intracellular platinum concentration due to a reduced influx, which was followed by reduced DNA platination [8].

Consequently, a circumvention of the reduced influx should result in a diminution of resistance [13,14]. In both the ovarian carcinoma and the ileocecal colorectal adenocarcinoma cell line pair **7** was able to bypass the reduced influx in the resistant cell line. To estimate the impact of the increased influx of **7** on DNA platination and resistance factor, the latter parameters were quantified and compared with those after oxaliplatin treatment (Table 3).

However, neither the reduced accumulation of oxaliplatin nor the increased accumulation of **7** affected the degree of DNA platination in A2780cis compared to A2780 cells in the expected way. Nevertheless, A2780cis cells developed resistance to both platinum complexes. Consequently, the extent of accumulation seems to play a minor role in resistance to oxaliplatin and its analogues. Therefore, a circumvention of resistance with more lipophilic complexes was not achievable in this cell line pair. Resistance might be either mediated by an increased platinum-DNA adduct tolerance or by mechanisms that occur independently of the formation of DNA adducts [44,45]. The reason for the different processing of cisplatin and oxaliplatin might be the fact, that A2780cis cells obtained resistance after treatment with cisplatin. Thus, cisplatin-specific resistance mechanisms are not necessarily relevant in oxaliplatin resistance.

In contrast, in the ileocecal colorectal adenocarcinoma cell line pair the expected correlation between intracellular platinum concentration, DNA platination and resistance factor was found. In HCT-8ox cells a decreased platinum-DNA adduct formation was observed upon a decreased accumulation after treatment with oxaliplatin. However, other processes might be involved as the resistance factor was rather high. No significant repair of platinum-DNA adducts in both ileocecal colorectal adenocarcinoma cell lines was found (data not shown), thus it is entirely conceivable that enhanced adduct tolerance represented a second resistance mechanism in this cell line, which has often been detected in cells with acquired resistance to oxaliplatin [44]. A closer look to the relationship between the influx rate of **4**, **5** and **6** and the resistance factor showed, that despite their lower accumulation in HCT-8ox cells compared to their sensitive counterparts, the resistance factor diminished with increasing lipophilicity (Table 2). These results suggest that reduction of the resistance factor was either due to decreased inactivation of oxaliplatin analogues or mediated by mechanisms occurring after DNA binding. For instance, cellular processing of the platinum-DNA adducts generated by more lipophilic as well as bulkier carrier ligands might be altered in this cell line pair. Differences in the processing of cisplatin- and oxaliplatin-DNA adducts are well known, e.g. the failure of recognition of the more lipophilic and bulkier oxaliplatin-DNA adducts by proteins of the MMR system and other damage recognition proteins like HMG domain proteins [46–48]. Thus, an influence of the substituents of the DACH carrier ligand on cellular processing is conceivable.

Nevertheless, despite the advantage of a reduction in the resistance factor in HCT-8ox cells in the case of more lipophilic compounds, we failed to demonstrate overcoming resistance by these complexes because of their lower cytotoxicity compared to oxaliplatin. However, lipophilic platinum compounds represent an interesting starting point for further optimization studies. If these compounds could be delivered directly to their primary target, the DNA, the loss in cytotoxicity might be avoided.

Table 3
Contribution of cellular accumulation and DNA platination to resistance.

	A2780cis vs. A2780		HCT-8ox vs. HCT-8	
	Oxaliplatin	**7**	Oxaliplatin	**7**
Fold resistance	3.3	4.1	10.0	1.1
Fold decrease in accumulation after 2 h	1.6	0.8	1.6	0.8
Fold decrease in DNA platination	1.1	1.3	2.4	1.6

5. Conclusions

In conclusion, passive diffusion represented the main influx mechanism of oxaliplatin analogues in the early influx phase. The predominant influx mechanism in the late influx phase was lipophilicity-independent and might be mediated by transport proteins. Further investigations are needed to clarify the contribution of reactivity to influx and to identify the involved transport proteins.

More lipophilic compounds revealed a lower cytotoxicity, although the early influx rate was increased in the case of more lipophilic platinum complexes. Alterations in the reactivity of the platinum compounds with bulky substituents in the intracellular environment or increased sequestration of more lipophilic platinum complexes might be responsible for their reduced cytotoxicity in the investigated cell lines.

The resistance profiles of the two cell line pairs were found to be quite different, probably due to the different ways in acquiring resistance (cisplatin vs. oxaliplatin treatment). In the ovarian carcinoma cell line pair more lipophilic complexes had no advantage. In contrast, in the ileocecal colorectal adenocarcinoma cell line pair increasing lipophilicity led to a decrease in the resistance factor which does not seem to be related to increased influx. Decreased intracellular inactivation of platinum complexes, as well as different recognition and processing of the formed DNA adducts due to the variation of the carrier ligand provides possible explanations for this phenomenon which, however, requires further clarification. Nonetheless, due to the considerably lower cytotoxicity of more lipophilic compounds compared to oxaliplatin, overcoming resistance is not possible by enhancing lipophilicity.

Abbreviations

CTR1	copper transporter 1
IQR	interquartile range (measure of statistical dispersion being equal to the difference between the third and first quartile)
LLOQ	lower limit of quantification
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
OCT	organic cation transporter
OECD	Organisation for Economic Co-operation and Development
PBS	phosphate buffered saline
RF	resistance factor

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